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Full Length Research Paper

Heterogeneity of *Anopheles* Mosquitoes in Nyabushozi County, Kiruhura district, Uganda

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The diversity of *Anopheles* mosquitoes was studied for seven months in eight villages in Nyabushozi County, Kiruhura District, Uganda. The aim of the study was to identify sibling species of *Anopheles gambiae* S.L, and the *Anopheles funestus* group using ribosomal DNA polymerase chain reaction assays. A total of 2566 female *A. gambiae* S.L, and 270 *A. funestus* group mosquitoes were collected in 24 households using pyrethrum spray catches, and from goat pens and cattle housing (kraals), using CDC light traps. More *A. gambiae* S.L, and *A. funestus* were caught in goat's houses than in cattle kraals, probably due to the effect of weekly spraying of cattle. The densities of female *A. gambiae* S. L and *A. funestus* in all the eight villages studied were significantly influenced by rainfall. *A. gambiae* S.S. was the only sibling species within the *A. gambiae* complex identified by polymerase chain reaction assay. *A. funestus* was also identified by polymerase chain reaction and was found to be in sympathy with two other sibling species within the group, *A. nopheles leesoni* and *A. nopheles parensis*. Overall, *A. gambiae* S.S. was the most abundant species and probably contributed to most of the malaria transmission in Nyabushozi County, Kiruhura district.

Key words: *Anopheles* mosquitoes, heterogeneity, polymerase chain reaction, kiruhura district, Uganda.

INTRODUCTION

Malaria is the leading cause of morbidity and mortality in Uganda, especially in children under five years (Langi et al., 2001). It is estimated that over 90% of the population in Uganda lives in highly endemic areas with perennial transmission, while the remaining 10% live in low transmission areas that are prone to malaria epidemics. The Kiruhura district in southwest Uganda (formerly part of Mbarara district) lies towards the boundary of these two types of transmission and had low levels of malaria prevalence in the 1960's when a nationwide survey was carried out. More recently, the inhabitants have perceived a great increase in malaria incidence and ascribed this to changes in livestock management from nomadic pastoralism towards more settled farming. One hypothesis was that this malaria increase had been mediated by changes in feeding patterns of the primarily zoophilic, *Anopheles arabiensis* , a member of the *Anopheles gambiae* complex and the work were particularly directed to determining the role, if any, of *A. arabiensis* in relation to *A. gambiae* sensu strict (S.S.) in transmission of

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Table 1. Sample size of the target communities in the four sub-counties of Nyabushozi County, Kiruhura district.

Sub-county	Parish	Village	Total number of households in each village	Number of households sampled
Kenshunga	Nshwerenkye	Mugore	92	3
	Rushere	Akatongore	96	3
Nyakashashara	Nyakahita	Katooma	62	3
	Rurambira	Kakyeera	74	3
Sanga	Rwabarata	Kiribwa	113	3
	Nombe II	Ntuura	40	3
Kikatsi	Kanyanya	Ifura	58	3
	Kayonza	Rugaaga	55	3
Total			590	24

malaria under these particular conditions of environmental and livelihood change.

The most common vectors of malaria in Uganda are *A. gambiae* S.L. and *Anopheles funestus* group with *A. gambiae* S.L. being the dominant species in most places. *A. funestus* group are commonly found in high altitude areas and during the short dry seasons when permanent water bodies are the most common breeding sites (Uganda malaria control strategic plan 2005 - 2010, unpublished document). Mosquito species within the *A. funestus* group are closely related but morphologically indistinguishable. Also, the mosquito species within *A. gambiae* complex are closely related but morphologically indistinguishable. The distribution of *A. funestus* group, and *A. gambiae* complex in Uganda is not well understood, and information on their vectorial capacity remains fragmentary. This research work was aimed at identifying mosquito species in the *Anopheles* complexes occurring in Nyabushozi Country, at species level using molecular tools (ribosomal DNA polymerase chain reaction assay), so as to distinguish vector species from non-vectors, and establish the anthropophilic and zoophilic species. In addition, the study aimed at enhancing understanding of vector dynamics and the factors influencing their spatial and temporal distribution.

MATERIALS AND METHODS

Study area

This study was carried out in Nyabushozi county, Kiruhura district, south west Uganda, from October 2004 - April 2005. Nyabushozi county is located to the north and northeast of the Lake Mburo National Park within the Lake Victoria catchment, but lies in the rain shadow of the Kabula hills, which imparts a semi-arid type of climate. The average amount of rainfall in Nyabushozi County is 1000 mm per annum; its rainy seasons are March - May and September - November; dry spells are December - February and June - mid August. Current statistics (UBOS, 2005) give a figure of 100,630 people in Nyabushozi according to the 2002 Population and Housing Census Main Report.

Nyabushozi county has seven subcounties: Kenshunga, Nyakashashara, Sanga, Kikatsi, Kinoni, Kashongi and Kanyareru, covering 647 square kilometers with a population of about 50,000 people. It has the largest cattle population in the district, dominated by the indigenous Ankole breeds. Since cattle keeping are a major activity, many farmers spray their cattle weekly with acaricides such as amitraz to control ticks. A number of farmers also apply synthetic pyrethriods such as deltamethrin on their cattle for the control of both ticks and tsetse flies.

The county has several communal valley dams/tanks in addition to each household having its own water facility for both animals and people. The vegetation in the county includes *Acacia* tree species interspersed with the grasses, *Themeda triandra*, *Loudetia kagarensis* , *Brachiara sp*, *Cynodon sp*. and *Cymbopogon afronadus*. A variety of wild large herbivores graze between the National Park and the study area. The households have mainly grass thatched, 'igloos', semi permanent and permanent houses.

Recently, the county started experiencing some changes in livelihood patterns from a pastoral to an agro-pastoral production system, which involved a shift from living in traditional huts (grass-thatched and mud walls), usually sited very close to kraals (animal housing) to living in permanent structures located far from animal housing.

Field surveys were conducted in four subcounties: Kenshunga, Nyakashashara, Sanga and Kikatsi, for seven months from October 2004 - April 2005. A collection of adult *Anopheles* mosquitoes were done twice during the rainy seasons that is, October - November and November - December 2004 (with a spell of dry season in December) and twice during the dry season that is, January to February 2005. Two villages from each subcounty were chosen purposively for sampling of mosquitoes (Table 1). From each village, three households (pastoralist, transition farmer and a settled farmer) were sampled throughout the study period except when circumstances required substitution of households.

Sampling and processing of adult mosquitoes

Collection of adult mosquitoes in households was done between 8 a.m - 12 noon. Latitude and longitude data were recorded for the sampled sites (household, cattle and goat housings) using a hand-held navigational system, Global Positioning System (GPS). Adult

mosquitoes were collected from the inside of selected households using pyrethrum spray collection (PSC) method (WHO, 1975). The number of people who slept in the house the previous night, types of houses, and use of indoor residual spraying were also noted.

For outdoor catches, Centre for Diseases Control and Prevention (CDC) light traps were installed in goat's houses and cattle kraals throughout the night between 6:00 p.m - 7:00 a.m. The traps were deployed in the same households on the same day they were sprayed. The collected mosquitoes were processed and identified according to Gillies and Coetzee (1987). Samples of *Anopheles* female mosquitoes were preserved individually in silica gel and stored at ambient room temperature at the field station. The preserved mosquitoes were later transferred to Livestock Health Research Institute, Tororo for definitive identification and Polymerase chain reaction (PCR) analysis.

The legs and wings of individual adult mosquitoes were plucked out and put in separate labeled tubes. PCR amplification of DNA from legs and wings was performed for the *A. gambiae* complex as described by Scott et al. (1993) and for members of the *A. funestus* group as described by Koekemoer et al. (2002).

Primers used were specific for *A. gambiae* S.S and *A. arabiensis*, and the universal primer for the *A. gambiae* complex. Other specific primers targeting other members of the *A. gambiae* complex were not used because their natural habitats are not similar to the one found in Nyabushozi county. *Anopheles merus* are found in coastal areas of east Africa and breed in salt marshes, *Anopheles melas* are found in west Africa, *Anopheles bwambae* are found in mineral springs in western Uganda while *Anopheles quadriannulatus* (Theobald) species A is found in south Africa, and species B in Ethiopia (Hunt et al., 1998; Coetzee et al., 2000).

Primers for *A. funestus, Anopheles leesoni, Anopheles parensis, and Anopheles rivulorum* were also used for detection of the species within *A. funestus* group (Koekemoer et al., 2002) and the universal primer for the *A. funestus* group was also used. The primers were obtained commercially from Sigma-Genosys, United Kingdom.

Polymerase chain reaction assay for the *Anopheles gambiae* complex

Amplification was carried out in a total volume of 25 µl of reaction mixture containing 1.5 mM $MgCl_2$, 1× buffer, 200 µM each of dATP, dGTP, dTTP, dCTP, and 0.625 units of *Taq* DNA polymerase (Promega Corporation, Madison, USA) and sterile water. This mixture was overlaid with mineral oil and cycling was done as follows: a denaturation step at 94ºC for 5 min, followed by 30 cycles each at 94ºC for 30 s, 50ºC for 30 s, 72ºC for 30 s, with a final extension step at 72ºC for 10 min. Test samples and reagents were appropriately dispensed into each tube after which the tubes were capped and run in the GeneAmp PCR system 9700 PCR (PE Biostems Foster, California USA). *A. gambaie* PCR products were electrophoresed on 1.5% agarose gel (Sigma- Genosys, United Kingdom) under 5 µl of 5 mg/ml ethidium bromide (Sigma-Genosys, United Kingdom) and visualized on UV light.

Polymerase chain reaction assay for the *Anopheles funestus* group

Each mosquito leg or wing was homogenized in a sodium-Tris-edetic acid (EDTA) buffer (0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8.6) and incubated at 94ºC for 10 min. Debris was precipitated by centrifuging at 6000 g for 1 min and 1 µl of DNA was used for PCR analysis.

Amplification was carried out in a total volume of 25 µl of the reaction mixture containing 1.5 mM $MgCl_2$, 1X buffer, 200 µl each dATP, dGTP, dTTP, dCTP, and 0.625 units of *Taq* DNA polymerase (Promega Corporation, Madison, USA) and sufficient water. Amplification involved denaturation step at 94ºC for 2 min, followed by 30 cycles each at 94ºC for 30 s, 45ºC for 30 s, 72ºC for 40 s, with a final extension step at 72ºC for 5 min. Test samples and reagents were dispensed into each tube after which the tubes were capped and run in the PCR machine. *An. funestus* PCR products were electrophoresed on 2.5% agarose gel (Sigma-Genosys, United Kingdom) under 5 µl of 5 mg/ml ethidium bromide (Sigma-Genosys, United Kingdom) and visualized on UV light. Five microliters (5 µl) of the marker/DNA ladder, 10 µl of the known positive controls (known *Anopheles* mosquitoes samples obtained from Liverpool School of Tropical Medicine and tested together with all the collected field samples) and 10 µl of the PCR amplified product were loaded to respective well and electrophoresed at a constant voltage of 100 V. Visualized products were photographed using a polarized film camera and band sizes read (Figure 4).

RESULTS

Identification and abundance of vector species

A total of 24 households were sampled for *Anopheles* mosquitoes using pyrethrum spray collection method in eight villages in Nyabushozi county between October 2004 - April 2005. The total numbers of females of the *A. gambiae* complex and *A. funestus* group mosquitoes caught in indoor spraying in the four rounds of surveys, in all the eight villages, were 1544, and 186, respectively (Table 2). A total of 430 *A. gambiae* S.L. and 164 *A. funestus* group males were also caught during indoor spraying. Moreover, some Culicines (n = 12), *Mansonia* sp. (n = 3) and *Aedes aegypti* (n = 1) were also collected during this study.

The average numbers of *A. gambiae* S.L. females caught per night varied greatly among the eight villages, ranging from 38.3 - 105.3 females per household. Mugore village recorded the highest average number of 105. 3 females *A. gambiae* S.L. mosquitoes per house-hold while Rugaaga had the lowest catch of 38.3 females per household (Figure 3). *A. funestus* group were only caught in Mugore, Kiribwa and Kakyeera villages. The average number of female *A. funestus* group caught per night per household was highest in Kakyeera with 52.3 female mosquitoes and lowest catch was recorded in Mugore with 2.2 females *A. funestus* group per household (Figure 3).

Looking at the different surveys, the number of mosquitoes collected showed seasonal variations, with the highest catch of *A. gambiae* S. L. and *A. funestus* group females being recorded in the wet season or soon after the rains (Table 2). Mugore village had the highest female *A. gambiae* S.L. caught in the second survey when compared to other villages, while Kakyeera village recorded the highest female *A. funestus* group catches throughout the study (Table 2).

Light trap collection in cattle kraals and goat houses A

total of 3 *A. gambiae* S.L. females were caught in cattle

Table 2. Female *Anopheles gambiae* and *Anopheles funestus* mosquitoes caught in each round of malaria vector survey.

Village	Houses sampled	1st Survey (Oct-Nov 2004) (beginning of main wet season)		2nd Survey (Nov-Dec 2004) (end of main wet season)		3rd Survey (Jan-Feb 2005) (main dry season)		4th Survey (April 2005) (beginning of short rain season)	
		A. gambiae	*A. funestus*	*A. gambiae*	*A. funestus*	*A. gambiae*	*A. funestus*	*A. gambiae*	*A. funestus*
Mugore	3	66	0	205	0	21	0	24	6
Akatongore	3	13	0	73	0	8	0	48	0
Katooma	3	26	0	77	0	3	0	56	0
Kakyeera	3	54	89	70	16	2	38	27	14
Kiribwa	3	77	0	82	2	7	0	81	21
Ntuura	3	40	0	30	0	36	0	98	0
Ifura	3	129	0	8	0	3	0	65	0
Rugaaga	3	59	0	4	0	12	0	40	0
Total		464	89	549	18	92	38	439	41

The population of female *A. gambiae* S.L and *A. funestus* group mosquitoes increased in rainy season between October - December. Mugore had the highest catches of *A. gambaie* S.L. mosquitoes while Kakyeera recorded highest female *A. funestus* group catches.

kraals and 19 from goat houses using CDC light traps in all the villages during the surveys (Table 3). One female *A. funestus* group mosquito was caught in a cattle kraal and 83 from goat houses. More of *A. funestus* group mosquitoes were caught in goat houses as compared to cattle kraals. A total of 353 female Culicines, 36 *Mansonia* sp. and 33 *Ae. aegypti* were caught in the cattle kraals and in the goat houses.

Anopheles species composition and their seasonal variations

The 223 samples of *A. gambiae* S.L. analyzed (representing 14.2% of total population) using PCR were identified as *A. gambiae* S.S. (n = 1544) (Figure 4). No *A. arabiensis* were found. In addition, from the 54 *A. funestus* group (n = 270) analyzed using PCR (constituting 20% of total population), 6 were *A. funestus*, 13 *A. leesoni* and 35 *A. parensis*.

Anopheles gambiae S.S. population showed seasonal variations, with the highest densities occurring in the wet season or soon after the rains (Figure 1) . The populations were however lower during the wet/dry season interphase that is, from November - December 2004, and dry season from January - February, 2005.

The other mosquito species registered low population densities during the four seasons. No significant differences were observed in the population of *A. parensis* and *A. leesoni* during the rainy and dry seasons (p = 0.445). However, only small numbers of *A. funestus* were identified during the wet season (October - November, 2004), and dry season (January - February, 2005) (Figure 1). Comparison of *Anopheles* species distribution across the villages showed *A. gambiae* S.S. to be predominant throughout the eight villages studied (Figure 2). Only few *A. funestus* were identified in Mugore, Akatongore, Ifura, Katooma and Kakyeera villages. Kakyeera village had the highest catch of *A. parensis*, while Mugore village had the highest catch of *A. leesoni* (Figure 2).

DISCUSSION

This study was conducted in Nyabushozi county, Kiruhura district, to identify available mosquito species, and to enhance understanding of vector dynamics and the factors influencing their spatial and temporal distribution. The results show that more of the female *A. gambiae* were caught per household during rainy season or at the beginning of the rains. This was attributed to many suitable mosquito breeding grounds created by the rainy season, in addition to many valley dams owned by the households in Nyabushozi county. More of *A. funestus* group was collected in Kakyeera villages than in other areas. Kakyeera village is located in a rain shadow area and is situated at the shores of Lake Kakyeera that provides permanent mosquito breeding ground throughout the year.

Table 3. CDC light trap catches of mosquitoes in cattle kraals and goat houses.

Mosquitoes	1st Survey (Oct-Nov 2004)		2nd Survey (Nov-Dec 2004)		3rd Survey (Jan-Feb 2005)		4th Survey (April 2005)	
	Cattle kraal	Goat's house	Cattle kraal	Goat's house	Cattle kraal	Goat's house	Cattle kraal	Goat's house
Female *An. gamiae*	1	15	1	0	1	0	0	4
Male *An. gambaie*	0	0	0	0	0	0	0	3
Female *An. funestus*	0	62	1	0	0	21	0	0
Male *An. funestus*	0	0	0	0	0	0	0	0
Female culicines	24	97	64	83	10	25	19	31
Male culicines	2	0	11	18	0	0	1	1
Mansoni	4	14	7	0	1	2	0	8
Aedes aegypti	4	14	6	0	2	6	0	1

Highest numbers of *A. funestus* group were caught in goat houses compared to cattle kraals. More female *A. gambiae* S.L. were caught in goat houses as compared to cattle kraals. Many culicines, mansonia and *Aedes* aegypti were also collected in cattle kraals and goat houses.

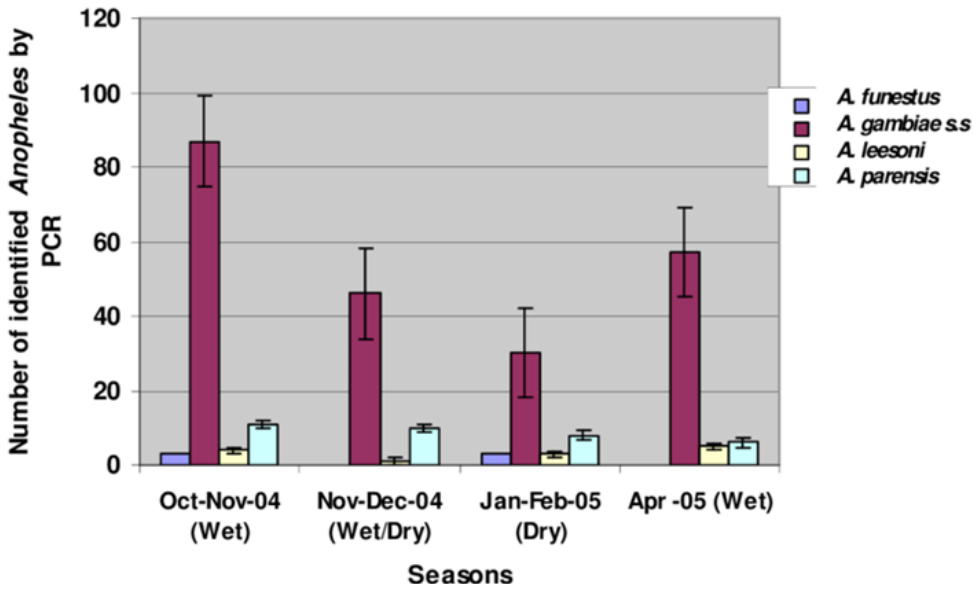

Figure 1. Seasonal comparison of Anopheles species distribution: There was seasonal variation in *Anopheles* species distribution with *A. gambiae* S.S showing highest catches in rainy season between October, November and April. The standard error bars indicate significant difference in *A. gambaie* S.S. population in October - November as compared to dry season January - February. The distribution of *A. leesoni*, *A. parensis* and *A. funestus* do not show significant variation across dry and rainy seasons.

A. funestus group breeds in the lake waters so it is predominant all the year round in this village. More A. gambiae S.L. and A. funestus group were also caught in the goat houses than the cattle kraals. This may be attributed to the practice of the community weekly spraying of cattle with acaricides, which could deter mosquitoes from feeding on them. The goats were not sprayed with acaricides and could have served as alternative hosts. The catches of mosquitoes during this study could have been affected by variation in time used for PSC collection. The recommended time for PSC collection by WHO (1975) is between 6 a.m - 10 a.m. However, during this study period, mosquito collections could sometimes go up to midday due to the distance traveled to the study villages from the project field station and this could have allowed more mosquitoes to escape outside. The A. gambiae S.L. mosquitoes which were tested were identified as A. gambiae S.S., suggesting that this could be the only member of the A. gambiae complex in Nyabushozi Country, Kiruhura district. The absence of A. arabiensis which was suspected to be in this area

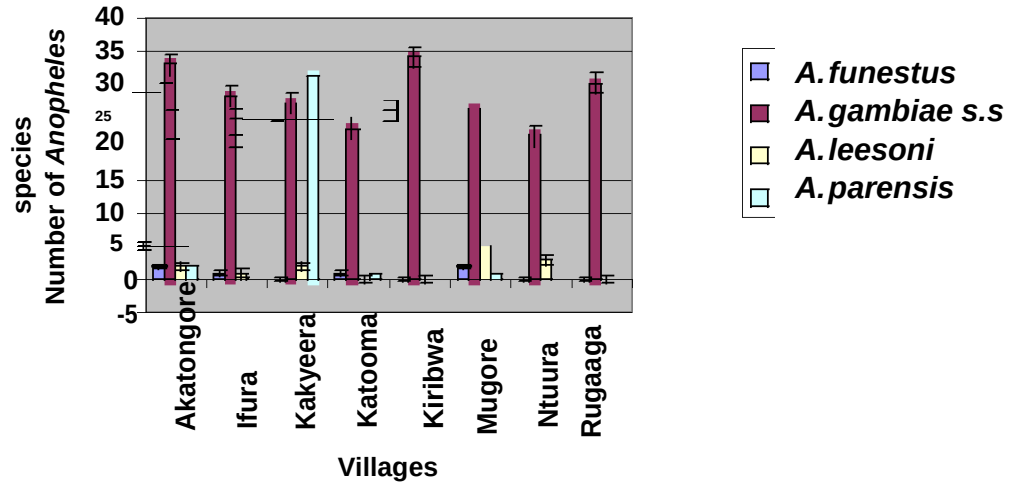

Figure 2. Comparison of *Anopheles* species across eight villages: *Anopheles gambaie* S.S. is the predominant *Anopheles* mosquito species in all the eight villages. Kakyeera village had the highest catch of *A. parensis* because of the permanent breeding areas provided by the shores of Lake Kakyeera.

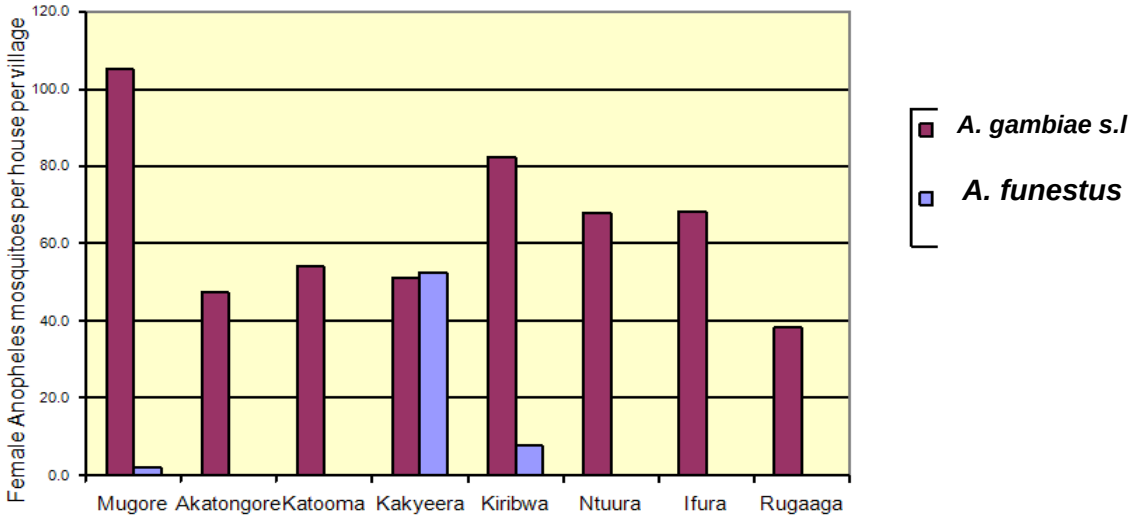

Figure 3. Comparison of female *Anopheles* mosquitoes per night across the eight villages in Nyabushozi country: *Anopheles gambaie* S.L. recorded highest number of catches per night across all the villages.

could be attributed to unsuitable environmental conditions for their breeding in the area. It is also possible that this sibling species could have been decimated due to intensive application of synthetic pyrethriod acaricides on cattle (weekly or fortnightly) for tick and tsetse control. The sample sizes were not huge and it is possible that *A. arabiensis* is present but at very low numbers. Increase in the population of *A. gambiae S.S.* was observed to coincide with rainy seasons.

With the aid of PCR, three species of the *A. funestus* group (*A. funestus*, *A. leesoni* and *An. parensis*) were identified, with *A. funestus* being the most anthropophilic and endophilic member of the group and highly efficient vector of malaria. *A. funestus* was found to be in

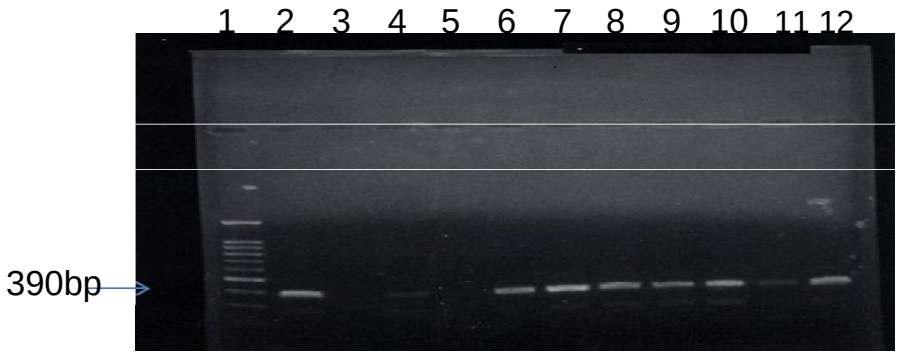

Figure 4. 1.5% agarose gel electrophoresis of *Anopheles gambiae PCR* products. Lane 1: Molecular marker, Lanes in mosquito samples amplified by rDNA primers. Lane 2: Positive controls. Lane 3: Negative control. Lane 4, 5, 6, 7.8,9,9,10,11, and 12 are products of *Anopheles gambiae* S.S.

sympatry with the other members of the group that included *A. leesoni* and *A. parensis* and this agrees with the earlier findings of Gilles et al. (1968 and 1987). Most of the *A. parensis* were caught in the goat houses. This is similar to the findings of Gilles et al. (1968).

Conclusions

The findings in the present study provides useful information on the mosquito species composition in Nyabushozi county, Kiruhura district, which can be a basis for formulating appropriate malaria control interventions in the area. Since *A. gambiae* S.S. and *A. funestus* identified in the study area are known to be highly endophagic and endophilic (feed and rest indoor), the use of insecticide treated nets (ITNs) and indoor residual spray (IRS) may be the appropriate vector con0trol strategies in the study area. In addition to IRS and ITNs, environmental management strategies present another potential intervention option that may be advocated to all household members in the area.

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